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AMPK and TBC1D1 Regulate Muscle Glucose Uptake After, but Not During, Exercise and Contraction

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Exercise increases glucose uptake in skeletal muscle independently of insulin signaling. This makes exercise an effective stimulus to increase glucose uptake in insulin-resistant skeletal muscle. AMPK has been suggested to regulate muscle glucose uptake during exercise/contraction, but findings from studies of various AMPK transgenic animals have not reached consensus on this matter. Comparing methods used in these studies reveals a hitherto unappreciated difference between those studies reporting a role of AMPK and those that do not. This led us to test the hypothesis that AMPK and downstream target TBC1D1 are involved in regulating muscle glucose uptake in the immediate period after exercise/contraction but not during exercise/contraction. Here we demonstrate that glucose uptake during exercise/contraction was not compromised in AMPK-deficient skeletal muscle, whereas reversal of glucose uptake toward resting levels after exercise/contraction was markedly faster in AMPK-deficient muscle compared with wild-type muscle. Moreover, muscle glucose uptake after contraction was positively associated with phosphorylation of TBC1D1, and skeletal muscle from TBC1D1-deficient mice displayed impaired glucose uptake after contraction. These findings reconcile previous observed discrepancies and redefine the role of AMPK activation during exercise/contraction as being important for maintaining glucose permeability in skeletal muscle in the period after, but not during, exercise/contraction.

To meet the increased energy demand in skeletal muscle during exercise and contraction, glucose uptake is

markedly increased. After the cessation of exercise and contraction, energy demand decreases and muscle glucose uptake gradually returns to resting levels (1–5). Studies have shown that exercise/contraction uses mechanisms to increase muscle glucose uptake that are independent of those engaged upon insulin stimulation (6–8). It has been speculated that cellular feed-forward events secure the initial rise in glucose uptake upon initiation of exercise/contraction, whereas other events control glucose uptake during exercise/contraction based on cellular feedback signals (9). Along these lines, the reversal of glucose uptake after cessation of exercise/contraction is likely also a regulated process securing adequate cellular fuel supply in recovery. However, the nature of this process is still only weakly understood.

Skeletal muscle glucose uptake can be defined as a three-step process involving delivery, transport, and metabolism of glucose (10,11). During exercise, the glucose transport capacity is mainly determined by the representation of GLUT4 at the cell surface membrane that is regulated by both exo- and endocytotic processes (12). It is well known that pharmacological activation of the cellular energy sensor AMPK is sufficient to increase glucose uptake and GLUT4 representation at the cell surface membrane of skeletal muscle (13). An obvious extrapolation of these observations has been to assume that the AMPK activation seen in skeletal muscle during exercise/contractile activity (14,15) is regulating glucose uptake under these conditions as well. However, despite numerous studies, no consensus on the role of AMPK in regulating muscle glucose uptake during exercise/contractile activity has been reached (16).

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Going through the literature, we observed an unappreciated methodological difference between those studies reporting impairments in exercise/contraction-induced muscle glucose uptake by ablation of AMPK activity in skeletal muscle and those studies that did not. In the majority of studies reporting decreased muscle glucose uptake with contraction, the actual assessment of glucose uptake by muscle tracer accumulation took place either in the period during as well as in the period after contraction (17,18) or solely in the period after contraction (17,19–23). Moreover, studies have suggested that pharmacological agents activating AMPK increase muscle glucose uptake by slowing down GLUT4 endocytosis, whereas insulin and contraction increases glucose uptake by enhancing GLUT4 exocytosis (24–26). Inspired by these observations, we tested the hypothesis that the increase in glucose uptake during exercise/contractile activity occurs independently of AMPK, whereas AMPK becomes key for maintaining muscle glucose uptake in the period after exercise/contractile activity. Herein, using both conventional and conditional AMPK α 1 α 2 muscle-specific double-knockout (mdKO) mice as well as whole-body TBC1D1 KO mice, we demonstrate that AMPK is not necessary for contraction to increase muscle glucose uptake. In contrast, we provide evidence to support that AMPK regulates muscle glucose uptake in the immediate period after exercise/contraction and that this is likely mediated by phosphorylation of its downstream target TBC1D1. Collectively, these results redefine the biological role of AMPK and TBC1D1 signaling in the context of skeletal muscle glucose uptake.

RESEARCH DESIGN AND METHODS

Animals

All animal experiments were approved by the Danish Animal Experiments Inspectorate and complied with the EU convention for the protection of vertebrates used for scientific purposes. Animals used in this study were conventional and conditional (inducible) AMPK α 1 α 2 mdKO and recombinant congenic TBC1D1-deficient (whole-body TBC1D1 KO) female mice with corresponding wild-type (WT) littermates as controls (27–29). Mice (16 \pm 2 weeks [mean \pm SD]) were group housed at two animal facilities at the Department of Experimental Medicine and kept in a temperature- and humidity-controlled room on a 12:12 h light-dark cycle with free access to standard rodent chow and water.

Muscle Glucose Uptake After Exercise

All mice were acclimatized to treadmill running and subsequently subjected to a graded maximal running test as previously described (30). For muscle glucose uptake measurements after exercise, mice were fasted in single cages for 2 h before either performing a single bout of treadmill exercise (30 min, 10° incline, and 60% of maximal running speed) or resting as sedentary controls. Immediately after rest/exercise, mice were anesthetized by an intraperitoneal

injection of pentobarbital (9 mg/100 g body weight) and left to recover on a heating plate (30°C) for 30 min. Hereafter, a bolus of [3 H]2-deoxyglucose (12.3 MBq/kg body weight) dissolved in isotonic saline was administered retroorbitally. After 10 min, during which blood sampling was performed at time points 0, 5, and 10 min, tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were rapidly dissected and frozen for analysis of muscle glucose uptake as previously described (31).

Muscle Glucose Uptake During and After In Situ Contraction

Glucose uptake in TA and EDL muscles during in situ contraction was determined as previously described (31). In short, an electrode was placed on the common peroneal nerve of one leg of anesthetized animals (intraperitoneal injection of pentobarbital, 9 mg/100 g body weight), after which electrical stimulation was applied for 10 min to initiate contraction of the TA and EDL muscles. The other leg served as a sham-operated control. Muscle glucose uptake during in situ contraction was determined by retro-orbital injection of [3 H]2-deoxyglucose as described above. For muscle glucose uptake measurements after in situ contraction, anesthetized and muscle-contracted animals were left to recover on a heating plate (30°C) for 30 or 60 min, after which muscle glucose uptake was determined as described above.

Insulin-Stimulated Glucose Uptake of Incubated Skeletal Muscle

Fed animals were anesthetized by an intraperitoneal injection of pentobarbital (10 mg/100 g body weight) before EDL muscles were isolated and suspended in incubation chambers containing Krebs-Ringer buffer (KRB) as previously described (31). In short, EDL muscles were incubated for 30 min in the absence or presence of 100 μ U/mL insulin, after which half of the muscles were transferred to new incubation chambers containing KRB for either 30 min or 1 h. 2-Deoxyglucose uptake was measured during a 10-min period after the respective incubation period by adding 1 mmol/L [3 H]2-deoxyglucose (0.028 MBq/mL) and 7 mmol/L [14 C]mannitol (0.0083 MBq/mL) to the incubation medium. 2-Deoxyglucose uptake was determined as previously described (31).

Glucose Uptake in Incubated Skeletal Muscle 2 h After Prior In Situ Contraction

After in situ contraction of a single mouse hind limb, EDL muscles from both legs were isolated and incubated in incubation chambers with KRB as described above. 2-Deoxyglucose uptake was measured during the last 10 min of the 2-h incubation period by adding 1 mmol/L [3 H]2-deoxyglucose (0.028 MBq/mL) and 7 mmol/L [14 C]mannitol (0.0083 MBq/mL) to the incubation medium. During the incubation period, KRB was replaced at time points 30, 60, and 90 min to minimize fluctuations in energy substrate content (pyruvate) of the KRB.

Muscle Processing, SDS-PAGE, and Western Blot Analyses

Muscles were homogenized as previously described (31,32), and lysates were collected and frozen in liquid nitrogen for subsequent analyses. The bicinchoninic acid method was used to determine total protein abundance in muscle lysates. Lysates were boiled in Laemmli buffer and subjected to SDS-PAGE and immunoblotting as previously described (31,32).

AMPK Activity

Heterotrimer-specific AMPK activity in skeletal muscle was determined by three consecutive immunoprecipitations as previously described (31,32).

Muscle Glycogen and Glucose-6-phosphate

Muscle glycogen was measured on muscle protein homogenate after acid hydrolysis as previously described (31). Muscle glucose-6-phosphate content was measured fluorometrically as described by Lowry and Passonneau (33).

Antibodies

Primary antibodies against pAMPK α -Thr172 (2531), pACC-Ser79/212 (3661), pTBC1D4-Thr642 (8881), pErk1/2-Thr202/Tyr204 (9101), pP38MAPK-Thr180/Tyr182 (9211), Erk1/2 (9102), P38MAPK (9212), GAPDH (2118), and TBC1D1 (4629) were from Cell Signaling Technology. Antibodies against pTBC1D1-Ser231 (07-2268) and TBC1D4 (AS160) (07-741) were from Millipore, and AMPK α 2 (SC-19131), hexokinase II (SC-6512), and GLUT4 (PA1-1065) antibodies were from Santa Cruz Biotechnology and Thermo Fisher Scientific, respectively. ACC protein was detected using horseradish peroxidase-conjugated streptavidin from Dako (P0397). AMPK α 1 protein were detected using antibody as previously described (30,34). Antibodies used for the AMPK activity assay were raised against AMPK γ 3 (provided by D.G. Hardie, University of Dundee, Scotland, U.K.), AMPK α 2 (SC-19131; Santa Cruz Biotechnology), and AMPK α 1 (purchased from GenScript, Jiangning, Nanjing, China).

Statistical Analyses

Statistical analyses were performed using SigmaPlot (version 13.0; SYSTAT, Erkrath, Germany). Data are presented as the means \pm SEM unless stated otherwise. Two-way ANOVA with and without repeated measures was used to assess statistical differences. In case of unequal variance (Fig. 1E and G), the two-sided Student *t* test was used to assess statistical difference between groups. The Student-Newman-Keuls test was used for post hoc testing. Correlation analyses were performed by calculating the Pearson product moment correlation. Statistical significance was defined as $P < 0.05$.

RESULTS

Glucose Uptake Is Decreased in AMPK-Deficient Muscle in Recovery From Exercise and Contraction

We have previously shown that glucose uptake during in vivo exercise is intact in skeletal muscle from conventional AMPK α 1 α 2 mdKO mice (30). However, when evaluating muscle glucose uptake 30 min after in vivo exercise,

we found that glucose uptake was significantly lower in skeletal muscle from AMPK α 1 α 2 mdKO mice compared with WT littermates (Fig. 1A). We next sought to demonstrate whether reductions in muscle glucose uptake after in vivo exercise were imitated in a well-controlled experimental setup using nerve stimulation to elicit in situ muscle contraction in anesthetized mice. Again, we found that glucose uptake during in situ contraction was similar in muscle of WT and AMPK α 1 α 2 mdKO mice, whereas glucose uptake was significantly decreased in skeletal muscle from AMPK α 1 α 2 mdKO mice compared with WT mice 30 min and 1 h after contraction (Fig. 1B). We confirmed this phenotype in skeletal muscle of the inducible AMPK α 1 α 2 mdKO model (Fig. 1C and Supplementary Fig. 1A), ensuring that the phenotype observed in the conventional AMPK α 1 α 2 mdKO model is unlikely to be associated with secondary adaptations caused by embryonic AMPK α 1 α 2 deletion. The in situ contraction protocol reduced glycogen content (Fig. 1D and E) and increased phosphorylation of Erk1/2 Thr202/Tyr204 and p38 MAPK Thr180/Tyr182 (Fig. 1F–H) in skeletal muscle to an extent that did not differ between genotypes. This indicates that the in situ contraction protocol induced comparable changes in skeletal muscle from WT and AMPK-deficient mice. Importantly, we found that muscle glucose uptake during recovery from submaximal insulin stimulation was not affected by acute loss of AMPK activity in skeletal muscle (Supplementary Fig. 1B), demonstrating that the diminished ability of AMPK-deficient mice to maintain elevated glucose uptake rates during recovery is confined to the contraction stimulus.

AMPK-Deficient Mice Exhibit Intact Capacity for Delivery, Transport, and Phosphorylation of Glucose in Skeletal Muscle

Muscle glucose uptake has been shown to depend on delivery, transport, and phosphorylation of glucose (10). Delivery of glucose to the muscle is dependent on the blood glucose concentration as well as blood flow, and transport and phosphorylation of glucose is dependent on GLUT4 and HKII protein abundance/activity, respectively. When measuring blood glucose concentrations, we did not detect differences between genotypes during and in recovery from in situ contraction (Fig. 2A and Supplementary Fig. 2A). Moreover, we found that the muscle protein abundance of GLUT4 and HKII was not compromised in AMPK α 1 α 2 mdKO muscle (Fig. 2B, C, and F) but slightly decreased in inducible AMPK α 1 α 2 mdKO muscle compared with WT muscle (Supplementary Fig. 2B and C). Interestingly, measurements of the glucose metabolite glucose-6-phosphate, an allosteric inhibitor of HKII activity, revealed decreased levels in muscle from AMPK α 1 α 2 mdKO mice compared with WT mice in the period after contraction (Fig. 2D). We suspect this to be a consequence of the observed decrease in glucose uptake. Collectively, these data suggest that the capacity to take up glucose into muscle is similar between genotypes and that HKII activity

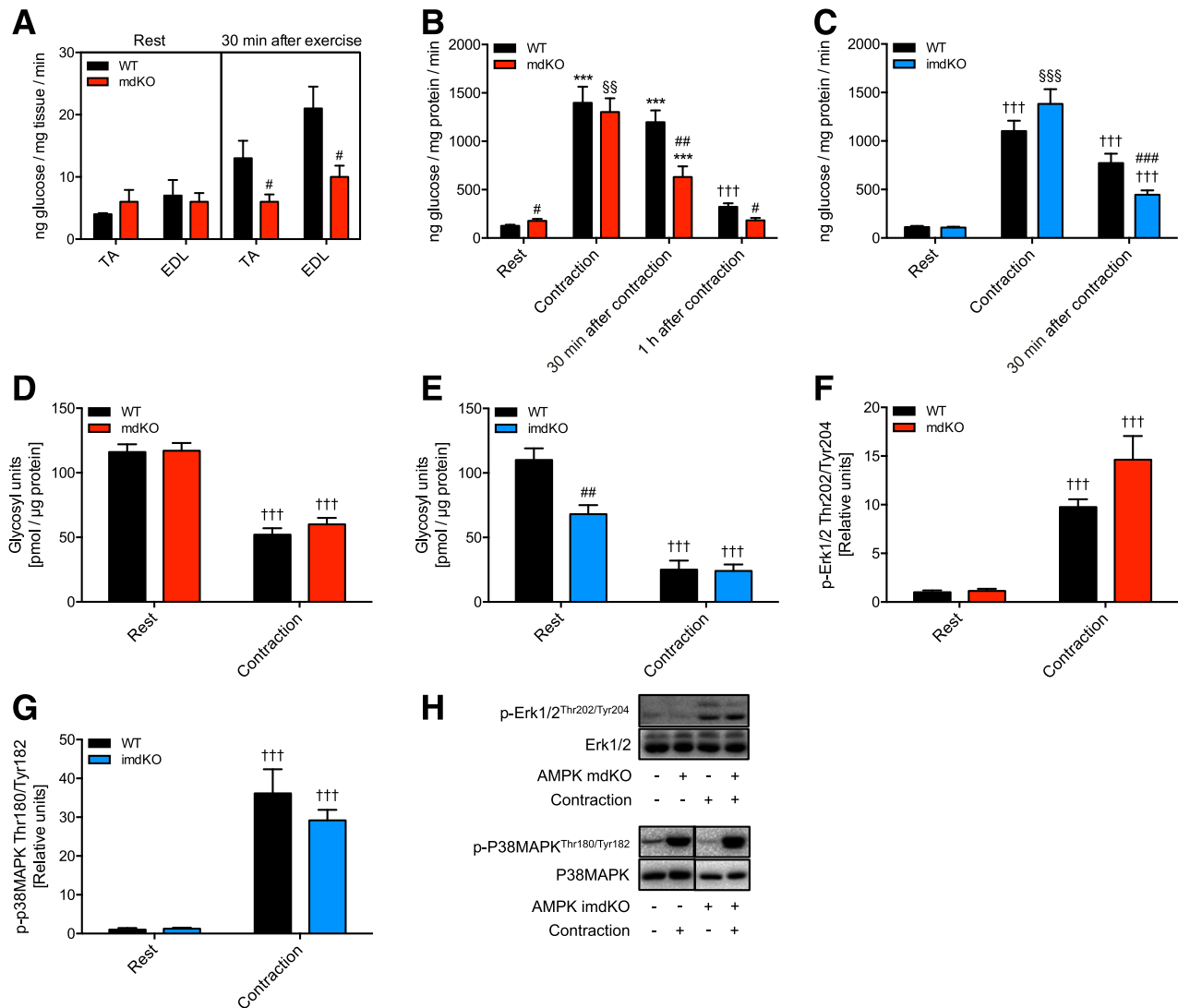


Figure 1—Glucose uptake in the period after exercise and contraction is decreased in AMPK-deficient skeletal muscle. **A:** In vivo glucose uptake in EDL and TA muscle from AMPK mdKO mice (red bars) and WT littermates (black bars) at rest and 30 min after treadmill exercise (rest, $n = 3$; exercise, $n = 6-9$). **B and C:** In vivo glucose uptake in TA muscle from AMPK mdKO mice, AMPK imdKO mice (blue bars), and WT littermates at rest, during in situ contraction, and 30 min and 1 h (only AMPK mdKO mice) after contraction (rest, $n = 9-23$; contraction, $n = 4-10$; 30 min after contraction, $n = 5-10$; 1 h after contraction, $n = 6$). **D and E:** Glycogen content in TA muscle from AMPK mdKO mice, AMPK imdKO mice, and WT littermates at rest and in response to 10 min in situ contraction (rest, $n = 9-20$; contraction, $n = 4-10$). **F and G:** Phosphorylation of Erk1/2 Thr202/Tyr204 (AMPK mdKO) and P38MAPK Thr180/Tyr182 (AMPK imdKO) in TA muscle from AMPK-deficient mice and WT littermates at rest and in response to 10 min in situ contraction (rest, $n = 9-20$; contraction, $n = 4-10$). **H:** Representative immunoblots. Significantly different from WT is indicated as follows: ### $P < 0.001$, ## $P < 0.01$, and # $P < 0.05$. Significantly different from rest and 1 h after contraction is indicated as follows: *** $P < 0.001$. Significantly different from rest, 30 min after contraction, and 1 h after contraction is indicated as follows: §§§ $P < 0.001$ and §§ $P < 0.01$. Significantly different from rest is indicated as follows: ††† $P < 0.001$. Statistical symbols presented in **A**, **D**, **F**, and **G** indicate main effect of intervention/genotype. Data in **A–G** are means \pm SEM.

and thus phosphorylation of glucose is likely not rate limiting for glucose uptake after contraction in AMPK-deficient muscle. To exclude that the reduced glucose uptake in AMPK-deficient muscle after in vivo exercise and in situ contraction was not due to a defect in glucose delivery, we measured glucose uptake in isolated and incubated EDL muscle from WT and AMPK α 1 α 2 mdKO mice. Compared with the rested muscle, we found that glucose uptake was still increased 2 h after in situ contraction in incubated muscle from WT mice, whereas

glucose uptake had returned to resting levels in prior contracted muscle from AMPK α 1 α 2 mdKO mice (Fig. 2E). Together this suggests that the mechanism responsible for maintaining elevated muscle glucose uptake after exercise/contraction is located at the intracellular level.

AMPK Activity in WT Muscle Reverses to Resting Levels 1 h After Contraction

Next we investigated whether the in situ contraction-induced increase in AMPK activity was maintained into

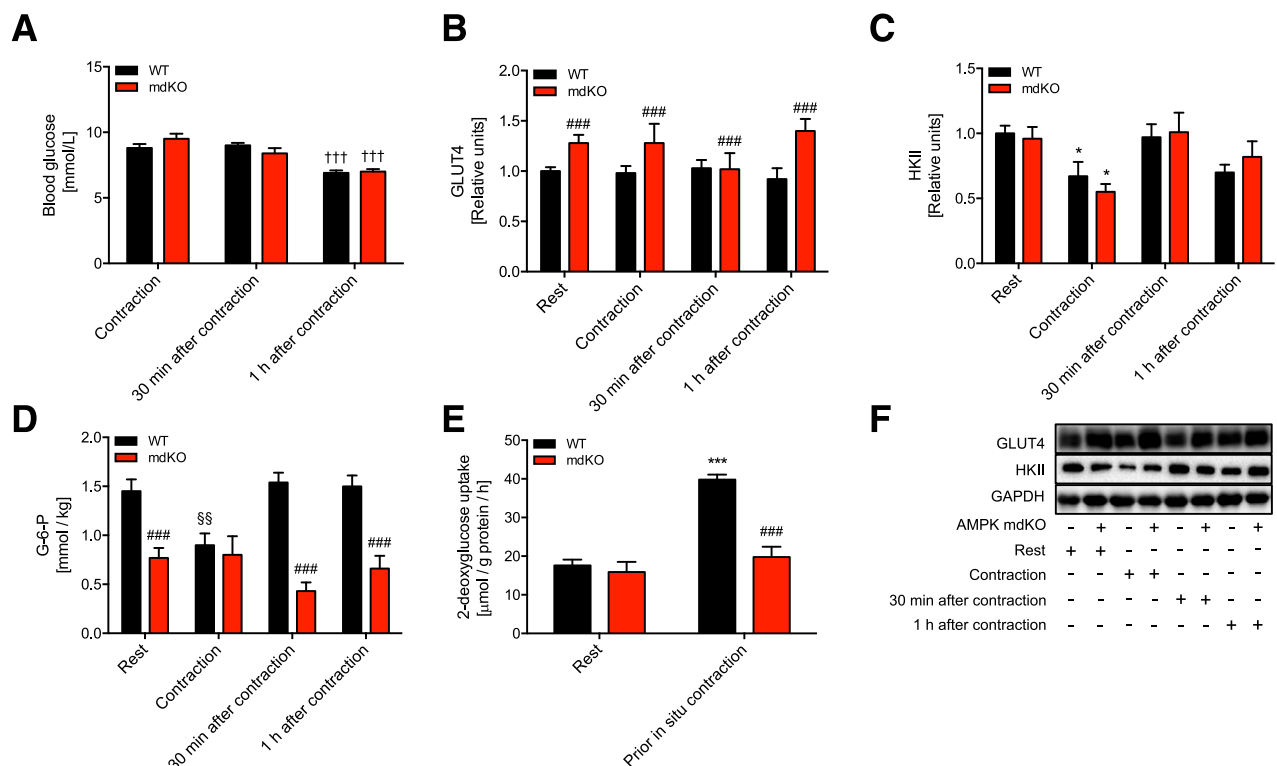


Figure 2—AMPK mdKO mice show normal capacity for glucose handling in skeletal muscle. **A:** Blood glucose concentration (mmol/L) in AMPK mdKO mice (red bars) and WT littermates (black bars) during, 30 min after, and 1 h after in situ contraction (contraction, $n = 15-21$; 30 min after contraction, $n = 24-30$; 1 h after contraction, $n = 18$). **B** and **C:** GLUT4 and HKII protein abundance in TA muscle from AMPK mdKO mice and WT littermates at rest, during in situ contraction, and 30 min and 1 h after contraction (rest, $n = 19-23$; contraction, $n = 5-7$; 30 min after contraction, $n = 8-10$; 1 h after contraction, $n = 6$). **D:** Glucose-6-phosphate (G-6-P) in TA muscle from AMPK mdKO mice and WT littermates at rest, during in situ contraction, and 30 min and 1 h after contraction ($n = 4-6$). **E:** Ex vivo glucose uptake in isolated and incubated EDL muscle from AMPK mdKO mice and WT littermates 2 h after rest or prior in situ contraction ($n = 3-4$). **F:** Representative immunoblots. Significantly different from WT is indicated as follows: ### $P < 0.001$. Significantly different from contraction and 30 min after contraction is indicated as follows: ††† $P < 0.001$. Significantly different from rest and 30 min after contraction is indicated as follows: *** $P < 0.001$ and * $P < 0.05$. Significantly different from rest, 30 min after contraction, and 1 h after contraction is indicated as follows: §§ $P < 0.01$. Statistical symbols presented in A–C indicate main effect of intervention/genotype. Data in A–E are means \pm SEM.

recovery, explaining greater glucose uptake in the period after contraction. We observed that the AMPK α 2 β 2 γ 3-, AMPK α 2 β γ 1-, and AMPK α 1 β γ 1-associated activity in WT muscle had returned to resting levels 1 h after contraction (Fig. 3A and Supplementary Fig. 3A and B). This was also mirrored by measurements of phosphorylated AMPK Thr172 and ACC Ser212 (Fig. 3B, C, and F and Supplementary Fig. 3C and D). Thus, although AMPK seems to be necessary for maintaining elevated muscle glucose uptake in recovery from exercise and contraction, the mismatch between AMPK activity and muscle glucose uptake 1 h after contraction indicates that one or several proteins regulated by AMPK and likely located closer to the GLUT4 recruitment event are responsible for maintaining higher rates of glucose uptake in skeletal muscle after exercise and contraction.

Glucose Uptake Is Decreased in TBC1D1-Deficient Muscle in Recovery From Contraction

Evidence indicates that AMPK-mediated glucose uptake involves phosphoregulation of TBC1D1, which has been

suggested to increase GLUT4 translocation to the muscle cell surface membrane (35). Interestingly, we found that phosphorylation of AMPK downstream target TBC1D1 Ser231 was increased 30 min and 1 h after in situ contraction (Fig. 3D–F). Moreover, these findings were positively correlated with glucose uptake in WT muscle but not in AMPK-deficient muscle (Fig. 3G and H and Supplementary Fig. 3E and F). In comparison, we did not observe associations between postcontraction glucose uptake and phosphorylation of Akt2 downstream target TBC1D4 Thr649 (Supplementary Fig. 3G and H). We also observed a small increase in phosphorylation of TBC1D1 Ser231 in skeletal muscle from AMPK-deficient mice after contraction. The reason for this is unclear, but it may be a consequence of AMPK-independent effects of contraction in muscle. Alternatively, contraction may lead to AMPK activation and thus phosphorylation of TBC1D1 in non-muscle cells that are present in the sample preparations of whole skeletal muscle tissue.

To support the possible role of an AMPK–TBC1D1 signaling axis regulating muscle glucose uptake after

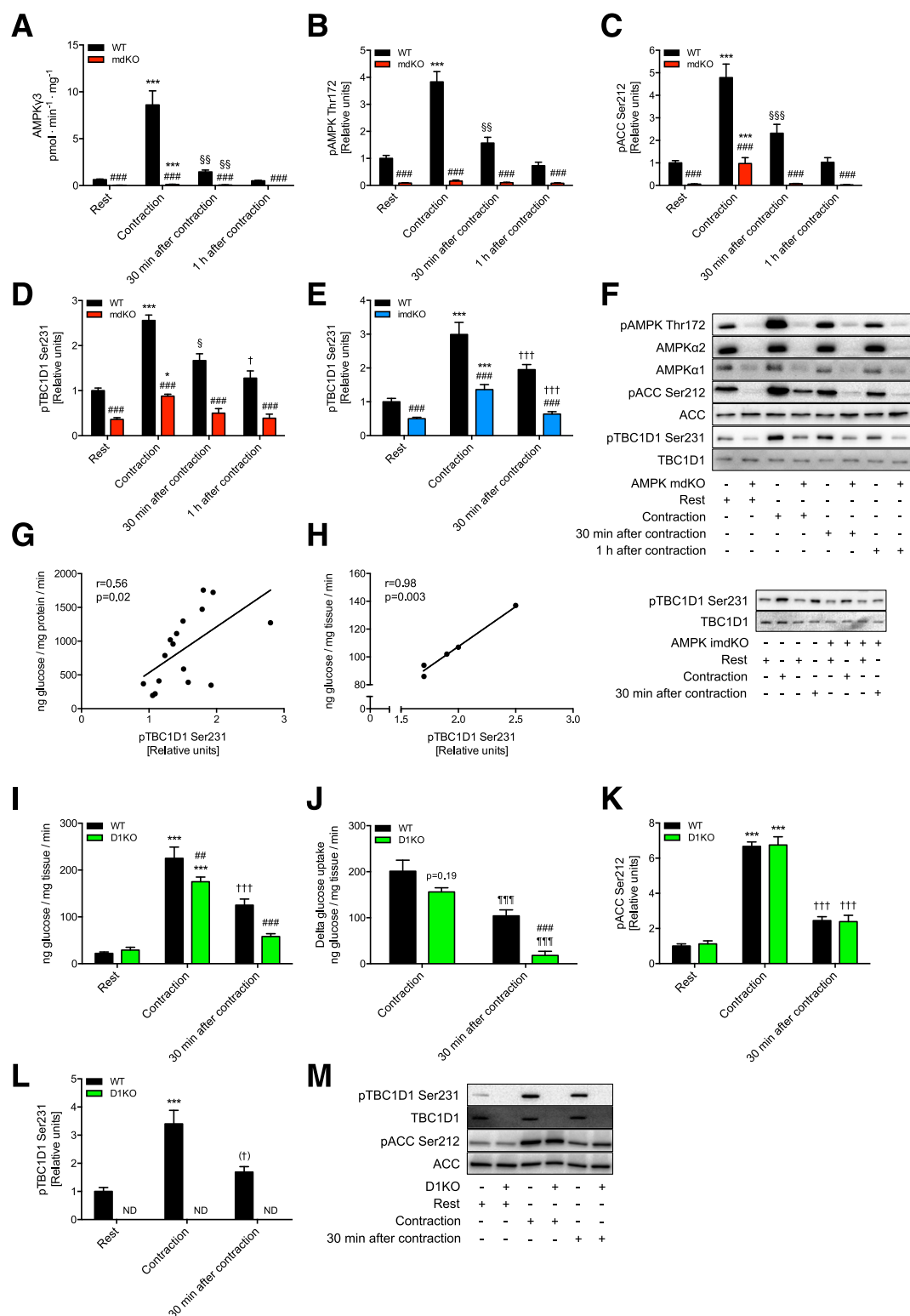


Figure 3—An AMPK-TBC1D1 signaling axis regulates muscle glucose uptake in the period after contraction. AMPK γ 3-associated activity (A) and phosphorylation of AMPK Thr172 (B), ACC Ser212 (C), and TBC1D1 Ser231 (D) in TA muscle from AMPK mdKO mice (red bars) and WT littermates (black bars) at rest, during in situ contraction, and 30 min and 1 h after contraction (rest, $n = 15$ –23; contraction, $n = 5$ –7; 30 min after contraction, $n = 4$ –10; 1 h after contraction, $n = 6$). E: Phosphorylation of TBC1D1 Ser231 in TA muscle from AMPK imdKO mice (blue bars) and WT littermates (black bars) at rest, during in situ contraction, and 30 min after contraction (rest, $n = 9$ –20; contraction, $n = 4$ –10; 30 min after contraction, $n = 5$ –10). F: Representative immunoblots for B–E. Pearson correlations between glucose uptake and phosphorylation of TBC1D1 Ser231 in TA muscle from WT littermates of AMPK mdKO (G) and AMPK imdKO mice (H) 30 min and 1 h after in situ contraction ($n = 16$ and $n = 6$, respectively). R and significance level are indicated in the respective panel. I: In vivo glucose uptake in TA muscle from TBC1D1 KO mice (green bars) and WT littermates (black bars) at rest, during in situ contraction, and 30 min after contraction (rest, $n = 11$ –12; contraction, $n = 6$; 30 min after contraction, $n = 5$ –6). J: The incremental increase (delta) in glucose uptake

contraction, we next investigated glucose uptake both during and after contraction in skeletal muscle from TBC1D1 KO mice. Compared with WT littermates, we found that the contraction-induced increase in glucose uptake reversed faster in skeletal muscle from TBC1D1 KO mice (Fig. 3I). This became even more apparent when calculating the incremental increase (delta values for individual paired muscles) in glucose uptake during and 30 min after contraction (Fig. 3J). Importantly, the contraction-induced increase in phosphorylation of ACC Ser212 was similar in skeletal muscle from both genotypes (Fig. 3K and M), indicating that KO of TBC1D1 does not compromise the ability of contraction to induce AMPK signaling in skeletal muscle. As expected, contraction only increased phosphorylation of TBC1D1 Ser231 in skeletal muscle from WT littermates (Fig. 3L and M). As previously reported (29), we found a ~50% reduction in GLUT4 protein abundance in skeletal muscle from TBC1D1 KO mice (Supplementary Fig. 3I), which likely accounts for the lesser increase in glucose uptake observed during *in situ* contraction (Fig. 3I).

The Majority of Studies Reporting Decreased Glucose Uptake in AMPK-Deficient Muscle During Exercise/Contractile Activity Measure Glucose Uptake in the Recovery Period

To the best of our knowledge, 19 studies, including the present one, have investigated glucose uptake in AMPK-deficient mouse skeletal muscle during exercise/contractile activity by radioactive glucose tracer accumulation. Intriguingly, 8 out of 10 studies reporting impaired contraction-induced glucose uptake in AMPK-deficient muscle actually measure glucose uptake in the period after contraction (Table 1). Such inconsistencies are also evident in studies reporting impaired contraction-induced glucose uptake in skeletal muscle from TBC1D1-deficient mouse models (Table 1). Collectively, these observations support the notion of an AMPK-TBC1D1 signaling axis regulating muscle glucose uptake in the period after exercise and contraction.

DISCUSSION

AMPK has long been known for its ability to regulate muscle glucose uptake based on numerous studies showing impaired glucose uptake in AMPK-deficient muscle when stimulated with pharmacological AMPK agonists (18,23,28,36,37). Since

AMPK is activated in skeletal muscle during exercise and contraction, common belief has been to assume that AMPK regulates muscle glucose uptake under these conditions as well. However, during the last 17 years, studies investigating the role of AMPK in regulating muscle glucose uptake during exercise and contraction have not been able to reach consensus.

To address this matter, we carefully examined whether the observed discrepancies in contraction-induced muscle glucose uptake between different AMPK-deficient mouse models were due to circumstances related to the timing of the uptake measurements. As a consequence, we performed measurements of muscle glucose uptake assessed by retroorbital injection of [³H]2-deoxyglucose during and after exercise/contraction in two mouse models with skeletal muscle AMPK deficiency. Using this approach, we now provide genetic evidence to support that AMPK regulates muscle glucose uptake after, rather than during, exercise/contraction. Based on these findings, we propose a model by which activation of AMPK in skeletal muscle during exercise acts to maintain a high glucose transport capacity in recovery from exercise. We propose that AMPK in this way secures a faster normalization of myocellular energy and fuel status after contractile activity.

As contraction acutely increases AMPK activity in skeletal muscle (14,31,38), we investigated whether this effect was maintained in recovery from contraction, potentially explaining our findings on glucose uptake. Interestingly, in WT skeletal muscle, we found that AMPK activity, as well as phosphorylation of AMPK Thr172 and ACC Ser212, had returned to resting levels 1 h after contraction, although glucose uptake was still increased compared with resting levels. In contrast, phosphorylation of the AMPK downstream target TBC1D1 was still increased 1 h after contraction, demonstrating that distinct signaling downstream of AMPK as well as glucose uptake are sustained for a long time during recovery even though AMPK activity is not. The reason for the apparent discrepancy between AMPK activity and downstream phosphorylation of TBC1D1 is unclear. We speculate that it relates to changes in phosphatase activity, altered cellular localization of TBC1D1, or time-based dynamics in protein phosphorylation. Studies in humans have also reported increased phosphorylation of TBC1D1 Ser231 in skeletal muscle as long as 30–180 min after the cessation of exercise (39,40). Interestingly, studies in rats and humans

(contraction/30 min after contraction values minus resting values) in TA muscle from TBC1D1 KO mice and WT littermates ($n = 5-6$). Phosphorylation of ACC Ser212 (K) and TBC1D1 Ser231 (L) in TA muscle from TBC1D1 KO mice and WT littermates at rest, during *in situ* contraction, and 30 min after contraction (rest, $n = 11-12$; contraction, $n = 6$; 30 min after contraction, $n = 5-6$). M: Representative immunoblots. Significantly different from WT is indicated as follows: ### $P < 0.001$ and ## $P < 0.01$. Significantly different from rest, 30 min after contraction, and 1 h after contraction is indicated as follows: *** $P < 0.001$ and * $P < 0.05$. Significantly different from rest and 1 h after contraction is indicated as follows: \$\$\$ $P < 0.001$, \$\$ $P < 0.01$, and \$ $P < 0.05$. Significantly different from rest is indicated as follows: ††† $P < 0.001$, †† $P < 0.05$, and (†) $P = 0.066$. Significantly different from contraction is indicated as follows: ¶¶¶ $P < 0.001$. Statistical symbols presented in A–C indicate main effect of intervention/genotype. Data in A–E and I–L are means \pm SEM. ND, not detectable.

Table 1—Overview of studies investigating exercise- and contraction-induced glucose uptake in AMPK- and TBC1D1-deficient mouse skeletal muscle by radioactive glucose tracer accumulation

Mouse model name	Target protein	Type of manipulation	Tissue specificity	Background strain	Exercise/contraction-induced glucose uptake or clearance			Tracer uptake measurement	Period of C: contractile activity, T: tracer exposure (min)	Reference
					Ex vivo	In situ	In vivo			
LKB1 KO	LKB1	Loss of protein	Heart and skeletal muscle (MCK)	C57Bl/6	↓EDL	—	—	After contraction	C: 0–10 T: 10–20	19
LKB1 KO	LKB1	Loss of protein	Heart and skeletal muscle (MCK)	C57Bl/6	—	↓EDL	—	After contraction	C: 0–5 T: 20–30	19
LKB1 MKO	LKB1	Loss of protein	Heart and skeletal muscle (MCK)	FVB	↓EDL	—	—	After contraction	C: 0–10 T: 10–20	20
α1 KO	AMPKα1	Loss of protein	Whole body	C57Bl/6-Sv129	↓SOL	—	—	After contraction	C: 0–10 T: 10–20	21
α1α2 imdKO	AMPKα1 and AMPKα2	Loss of protein	Skeletal muscle (HSA)	C57Bl/6-Sv129	—	↓(TA, EDL)	—	After contraction	C: 0–10 T: 40–50	This study
α2 KD	AMPKα2	Loss of activity	Heart and skeletal muscle (MCK)	C57Bl/6	↓SOL	—	—	After contraction	C: 0–10 T: 10–20	21
α2 KD	AMPKα2	Loss of activity	Heart and skeletal muscle (MCK)	C57Bl/6	↓EDL	—	—	After contraction	C: 0–2 T: 12–32	22
α2 KD	AMPKα2	Loss of activity	Heart and skeletal muscle (MCK)	C57Bl/6	↓EDL	—	—	After contraction	C: 0–10 T: 10–30	23
α2 KD	AMPKα2	Loss of activity	Heart and skeletal muscle (MCK)	C57Bl/6	—	↓(EDL, SOL)	—	After contraction	C: 0–15° T: 25–45	23
TBC1D1 Nob1.10 ^{S/L}	TBC1D1	Loss of protein	Whole body	C57Bl/6	↓EDL ¹ , ↔SOL	—	—	After contraction	C: 0–10 T: 20–40	63
TBC1D1 Nob1.10 ^{S/L}	TBC1D1	Loss of protein	Whole body	C57Bl/6	—	↑TA ¹	—	After contraction	C: 0–10 T: 40–50	This study
α2ITG	AMPKα2	Loss of activity	Heart and skeletal muscle (MCK)	FVB	↓EDL ²	—	—	After contraction	C: 0–10 T: 10–20	48
MLKB1KO	LKB1	Loss of protein	Heart and skeletal muscle (MCK)	FVB	↓SOL	—	—	After contraction	C: 0–10 T: 10–20	17
α1α2 mdKO	AMPKα1 and AMPKα2	Loss of protein	Skeletal muscle (HSA)	C57Bl/6-Sv129	—	↓TA	—	After contraction	C: 0–10 T: 40–50 and 70–80	This study
α2 KO	AMPKα2	Loss of protein	Whole body	C57Bl/6	↔SOL ²	—	—	After contraction	C: 0–10 T: 10–20	21
α2ITG	AMPKα2	Loss of activity	Heart and skeletal muscle (MCK)	FVB	↔EDL ²	—	—	After contraction	C: 0–10 T: 10–20	47
γ3 KO	AMPKγ3	Loss of protein	Whole body	C57Bl/6	↔EDL ²	—	—	After contraction	C: 0–10 T: 10–20	37
TBC1D1 Ser231Ala	TBC1D1	Loss of function	Whole body	C57Bl/6	↔EDL	—	—	After contraction	C: 0–10 T: 10–20	64
α1α2 mdKO	AMPKα1 and AMPKα2	Loss of protein	Skeletal muscle (HSA)	C57Bl/6-Sv129	—	—	↓(TA, EDL)	After exercise	C: 0–30 T: 60–70	This study
MLKB1KO	LKB1	Loss of protein	Heart and skeletal muscle (MCK)	FVB	—	↓TA	—	During and after contraction	C: 0–15 T: 0–45	17

Continued on p. 1435

Table 1—Continued

Table 1 —Continued										
Mouse model name	Target protein	Type of manipulation	Tissue specificity	Background strain	Exercise/contraction-induced glucose uptake or clearance			Tracer uptake measurement	Period of C: contractile activity, T: tracer exposure (min)	Reference
					Ex vivo	In situ	In vivo			
$\alpha 1$ KO	AMPK $\alpha 1$	Loss of protein	Whole body	C57Bl/6-Sv129	\leftrightarrow EDL, \downarrow SOL	—	—	During and after contraction	C: 0–10 T: 5–15	18
TBC1D1 4P mutant	TBC1D1	Loss of function	Skeletal muscle (OE by gene electrotransfer)	ICR	—	\downarrow TA	—	During and after contraction	C: 0–15 T: 0–45	45
TBC1D1 4A mutant	TBC1D1	Loss of function	Skeletal muscle (OE by gene electrotransfer)	ICR	—	\downarrow TA	—	During and after contraction	C: 0–15 T: 0–45	35
$\alpha 2$ ITG	AMPK $\alpha 2$	Loss of activity	Heart and skeletal muscle (MCK)	FVB	—	\leftrightarrow (EDL, TA, GAS) ²	—	During and after contraction	C: 0–15 T: 0–45	47
$\alpha 2$ KD	AMPK $\alpha 2$	Loss of activity	Heart and skeletal muscle (MCK)	C57Bl/6	\leftrightarrow (EDL, SOL)	—	—	During and after contraction	C: 0–10 T: 5–15	49
$\alpha 2$ KO	AMPK $\alpha 2$	Loss of protein	Whole body	C57Bl/6-Sv129	\leftrightarrow (EDL, SOL) ^{2,3}	—	—	During and after contraction	C: 0–10 T: 5–15	18
$\alpha 1\alpha 2$ imdKO	AMPK $\alpha 1$ and AMPK $\alpha 2$	Loss of protein	Skeletal muscle (HSA)	C57Bl/6-Sv129	—	\leftrightarrow (TA, EDL)	—	During contraction	C: 0–10 T: 0–10	This study
$\alpha 1\alpha 2$ mdKO	AMPK $\alpha 1$ and AMPK $\alpha 2$	Loss of protein	Skeletal muscle (HSA)	C57Bl/6-Sv129	\leftrightarrow (EDL, SOL) ³	—	—	During contraction	C: 0–10 T: 0–10	27
$\alpha 1\alpha 2$ mdKO	AMPK $\alpha 1$ and AMPK $\alpha 2$	Loss of protein	Skeletal muscle (HSA)	C57Bl/6-Sv129	—	\leftrightarrow TA	—	During contraction	C: 0–10 T: 0–10	This study
$\beta 2$ KO	AMPK $\beta 2$	Loss of protein	Whole body	C57Bl/6	\leftrightarrow EDL	—	—	During contraction	C: 0–20 T: 0–20	51
$\beta 2$ KO	AMPK $\beta 2$	Loss of protein	Whole body	C57Bl/6	\leftrightarrow EDL	—	—	During contraction	C: 0–20 T: 0–20	36
TBC1D1 <i>Nob1.10^{SL}</i>	TBC1D1	Loss of protein	Whole body	C57Bl/6	—	\leftrightarrow TA ^{1,3}	—	During contraction	C: 0–10 T: 0–10	This study
LKB1 MKO	LKB1	Loss of protein	Heart and skeletal muscle (MCK)	FVB	\leftrightarrow EDL	—	—	During contraction	C: 0–10 T: 0–10	20
$\beta 1\beta 2$ M-KO	AMPK $\beta 1$ and AMPK $\beta 2$	Loss of protein	Heart and skeletal muscle (MCK)	C57Bl/6	\downarrow (EDL, SOL) ⁴	—	—	During contraction	C: 0–10 T: 0–10	50
$\beta 1\beta 2$ M-KO	AMPK $\beta 1$ and AMPK $\beta 2$	Loss of protein	Heart and skeletal muscle (MCK)	C57Bl/6	\downarrow (EDL, SOL) ⁴	—	—	During contraction	C: 0–10 T: 0–10	51
$\alpha 2$ KD	AMPK $\alpha 2$	Loss of activity	Heart and skeletal muscle (MCK)	C57Bl/6	\downarrow (SOL, EDL) ⁷	—	—	During contraction	C: 0–10 T: 0–10	50
$\alpha 1\alpha 2$ mdKO	AMPK $\alpha 1$ and AMPK $\alpha 2$	Loss of protein	Skeletal muscle (HSA)	C57Bl/6-Sv129	—	—	\leftrightarrow (EDL, TA, GAS, SOL) ⁵	During exercise	C: 0–30 T: 0–30	30
$\alpha 2$ KD	AMPK $\alpha 2$	Loss of activity	Heart and skeletal muscle (MCK)	C57Bl/6	—	—	\leftrightarrow (QUAD, GAS)	During exercise	C: 0–30 T: 0–30	65
TBC1D1 KO	TBC1D1	Loss of protein	Whole body	C57Bl/6	—	—	\leftrightarrow R.QUAD, \downarrow W.QUAD ¹	During exercise	C: 0–20 T: 0–20	46

Continued on p. 1436

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Table 1—Continued

Mouse model name	Target protein	Type of manipulation	Tissue specificity	Background strain	Exercise/contraction-induced glucose uptake or clearance			Tracer uptake measurement	Period of C: contractile activity, T: tracer exposure (min)	Reference
					Ex vivo	In situ	In vivo			
TBC1D1 ^{Ser231Ala}	TBC1D1	Loss of function	Whole body	C57Bl/6	—	—	↔(QUAD, GAS)	During exercise	C: 0–35 T: 0–35	66
LKB1 MKO	LKB1	Loss of protein	Heart and skeletal muscle (MCK)	FVB	—	—	↔(QUAD, EDL)	During exercise	C: 0–20 T: 0–20	20
α2 KD	AMPKα2	Loss of activity	Heart and skeletal muscle (MCK)	C57Bl/6	—	—	↔GAS	During exercise	C: 0–20 T: 0–20	50
α2 KD	AMPKα2	Loss of activity	Heart and skeletal muscle (MCK)	C57Bl/6	—	—	↓(SOL, SVL, GAS) ⁶	During exercise	C: 0–30 T: 5–30	67
β1β2M-KO	AMPKβ1 and AMPKβ2	Loss of protein	Heart and skeletal muscle (MCK)	C57Bl/6	—	—	↓(SOL, GAS) ⁴	During exercise	C: 0–20 T: 0–20	51

The bold font indicates studies that support the hypothesis of AMPK being important for regulating muscle glucose uptake in the period after contraction and exercise. For some studies, information related to the study/model is important for the interpretation of data as this may help explain observed inconsistencies between defects in muscle glucose uptake and timing of uptake measurements. GAS, gastrocnemius; HSA, human skeletal actin; MCK, muscle creatine kinase; OE, overexpression; QUAD, quadriceps; R, red (oxidative); SOL, soleus; SVL, superficial vastus lateralis; KD, kinase dead; MKO, muscle-specific knockout; W, white (glycolytic). ¹These studies found decreased GLUT4 protein abundance in muscle from TBC1D1 KO mice compared with WT littermates that may account for the partial reduction in muscle glucose uptake observed during contraction and exercise. ²These studies reported that AMPKα1 activity and/or contraction-induced phosphorylation of ACC Ser212 were intact in skeletal muscle from the AMPK-deficient mice, which may preserve the ability to maintain elevated muscle glucose uptake in recovery from contraction. ³These studies showed that although the main effects of AMPK/TBC1D1 deficiency on muscle glucose uptake were present, the incremental increase (delta value) in contraction-induced muscle glucose uptake was not different between genotypes. ⁴This AMPK-deficient mouse model has drastically reduced mitochondrial content in skeletal muscle that may diminish muscle glucose uptake during contraction. ⁵This study reported that the exercise-induced glucose uptake in soleus muscle was significantly higher in the AMPK-deficient mouse model compared with WT littermates. However, this was likely due to elevated blood glucose concentrations in the AMPK-deficient mouse model during exercise. ⁶This study suggested that the observed decrease in glucose uptake/clearance in muscle from AMPK-deficient mice during exercise was likely due to impaired glucose delivery. ⁷These studies reported decreased force production in AMPK-deficient muscle compared with WT muscle that may account for the observed difference in muscle glucose uptake during contraction. ⁸This contraction protocol consisted of two 7-min in situ contraction periods with 1 min rest in between.

report that both phosphorylation of TBC1D1 and muscle glucose uptake return to pre-exercise levels 3–5 h in recovery from exercise (41–43). Collectively, these findings suggest that phosphorylation of TBC1D1 and glucose uptake decrease similarly in skeletal muscle after exercise but that these two measures may not always track with AMPK activity.

AMPK has been shown to target several proteins involved in regulating muscle glucose uptake, including TBC1D4 and PIKfyve (34,44). However, most evidence supports that AMPK-mediated phosphorylation of TBC1D1 (and not TBC1D4 and PIKfyve) facilitates AMPK-mediated effects on muscle glucose uptake (35,45). We found that phosphorylation of TBC1D1 is likely not important for regulating muscle glucose uptake during contraction, as phosphorylation of TBC1D1 was severely impaired in AMPK-deficient muscle even though contraction-induced glucose uptake was not. In contrast, we found that phosphorylation of TBC1D1 Ser231 in the period after contraction was positively associated with glucose uptake. Since this association was found in WT muscle only, this could indicate that AMPK phosphorylates TBC1D1 during exercise/contraction to secure muscle glucose uptake in the period after exercise/contraction. In support of this, we found that increased rates of glucose uptake in skeletal muscle after contraction were dependent on the presence of TBC1D1 as the contraction-induced increase in glucose uptake reversed faster in TBC1D1-deficient muscle compared with WT muscle. Because glycolytic skeletal muscle from TBC1D1 KO mice exhibits a loss in GLUT4 protein content (29), this likely accounts for the minor decrease in muscle glucose uptake observed during contraction. This is also supported by the observation that while exercise-induced glucose uptake is impaired in glycolytic quadriceps muscle from TBC1D1 KO mice, such a defect is not observed in oxidative quadriceps muscle, which expresses normal levels of GLUT4 protein content compared with muscle from WT littermates (46).

Going through the literature, we noticed that the majority of studies reporting impaired exercise/contraction-induced glucose uptake in AMPK-deficient muscle had unintentionally been measuring glucose uptake in recovery from exercise/contraction (Table 1), supporting the idea of AMPK being a regulator of muscle glucose uptake in the period after exercise/contraction. Nevertheless, some studies have reported intact contraction-induced glucose uptake in AMPK-deficient muscle although measurements of glucose uptake were performed in recovery (18,21,37,47–49), whereas others have reported impaired contraction-induced glucose uptake in AMPK-deficient muscle when measurements of glucose uptake were performed during contraction (50,51). These findings, however, may be related to incomplete KO of AMPK activity in skeletal muscle as KO of single catalytic subunits of AMPK has been shown to increase the activity of the remaining catalytic subunit during contractile activity (14). Also, secondary adaptations leading, for example, to reduced force production and reduced mitochondrial content in skeletal

muscle from AMPK-deficient mice may have masked the likely function of AMPK in regulating muscle glucose uptake after contraction (Table 1). We cannot ultimately rule out that AMPK may be necessary for regulating muscle glucose uptake during longer periods of exercise (>30 min) and contraction (>10 min) where the myocellular stress may be further elevated. However, measurements of skeletal muscle glucose uptake in AMPK kinase-dead mice and AMPK α 1 α 2 mdKO mice during longer periods of contraction (20 min) (52) and in vivo exercise (90 min) (J.R. Hingst, J.F.P.W., unpublished observations), respectively, argue against this idea.

Only a few studies have investigated whether TBC1D1 may be involved in regulating exercise/contraction-induced glucose uptake in skeletal muscle. Interestingly, two studies using gene electrotransfer into mouse TA muscle leading to overexpression of TBC1D1 mutated at several predicted AMPK phosphorylation sites report a 22% and 42% reduction in contraction-stimulated glucose uptake without a change in GLUT4 protein abundance between muscles transfected with control or mutated TBC1D1 (35,45). However, in both studies, muscle glucose uptake rates were measured as the combined uptake of glucose during (15 min) and after (30 min) contraction. Based on the findings presented here, this may suggest that the observed impairment in contraction-induced glucose uptake in skeletal muscle transfected with mutated TBC1D1 was due to measurements of glucose uptake rates in the recovery period from contraction.

If we permit translation of our observations in mice to human physiology, this may in fact bring some new aspects into former human observations. First, it has been shown that the activation of AMPK in human skeletal muscle in response to exercise (53–55) is not reversed immediately after exercise but is maintained above basal levels for a prolonged period of time. In some studies, this is evident from enhanced AMPK activity per se (40,54,56) and in other studies from enhanced AMPK downstream signaling (57,58). Thus, it would be expected that in muscle of humans, the sarcolemmal glucose transport capacity is maintained high in the period after exercise. Apparently this view conflicts with the observation that muscle glucose uptake quickly reverses to resting levels shortly after exercise (3,59). However, the reversal of glucose uptake is likely the result of a marked decrease in muscle perfusion (delivery) (60) rather than a decrease in sarcolemmal glucose permeability. This interpretation may be inferred from a study in which the muscular interstitial glucose concentration was measured in the period after exercise. Thus, several hours into recovery from one-legged exercise, the interstitial muscle glucose concentration was markedly lower in the prior exercised leg (~ 1.85 mmol/L) compared with the rested leg (~ 4.13 mmol/L) despite comparable blood flow to the two legs (61). Therefore we now propose that the maintained membrane glucose permeability in human skeletal muscle relates to elevated AMPK activity and downstream signaling in the recovery period from exercise. The enhanced glucose

permeability is thus favoring glucose uptake in previously exercised muscle once delivery of glucose (perfusion/plasma concentration) increases, for example, upon food intake and subsequent insulin release.

In conclusion, by measurements of muscle glucose uptake during and in recovery from exercise/contraction and careful evaluation of the literature, our findings provide new means of AMPK for the regulation of muscle glucose uptake. Our study seems to explain the large discrepancy that occurred in the aftermath of the seminal observation by Mu et al. (23) in 2001, indicating a role of AMPK in regulating muscle glucose uptake during contractile activity. Hence, it appears that the acute activation of AMPK in skeletal muscle during exercise/contraction acts to secure glucose uptake when the contractile activity ceases. This provides a mechanism by which a high glucose transport capacity is secured, favoring replenishment of muscle cellular energy stores after exercise/contraction. An important premise for this interpretation, if indeed AMPK functions to delay GLUT4 endocytosis in skeletal muscle (24), is that the stimuli leading to enhanced GLUT4 exocytosis prevail over GLUT4 endocytosis in regulating GLUT4 content at the cell surface membrane during exercise and contraction where AMPK activity is also elevated. Although debatable, such stimuli may include entry of extracellular Ca^{2+} as well as mechanical stress signaling (11,62). Together with our previous findings, revealing a role of AMPK in regulating muscle insulin sensitivity after exercise and contraction (31), the current study identifies AMPK as an important regulator of muscle glucose metabolism after, rather than during, exercise/contraction.

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Author Contributions. R.K. conceived and designed the research, performed the experiments, analyzed the data, and wrote the manuscript. J.L.W.R., N.O.J., and J.B.B. performed experiments and analyzed the data. M.F., B.V., A.C., and H.A.-H. provided founder mice for the study. J.F.P.W. conceived and designed the research and contributed to drafting the manuscript. All authors interpreted the results, contributed to the discussion, edited and revised the manuscript, and read and approved the final version of the manuscript. J.F.P.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data Availability. The data and resources generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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